



# HER-2, EGFR, Cox-2 and Ki67 expression in lymph node metastasis of canine mammary carcinomas: Association with clinical-pathological parameters and overall survival



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## ABSTRACT

Studies about canine mammary tumors based on single molecular markers probably cannot accurately account for the heterogeneity of this disease, and the investigation of multiple molecular alterations in primary tumors and their metastases, in conjunction, has assumed great importance for the understanding of mammary tumor progression. In the present study, we selected 54 primary mammary carcinomas with lymph node metastasis (T1,2,3N1M0), 29 primary mammary carcinomas without metastasis (T1,2,3N0M0), and 25 canine lymph nodes metastasis to evaluate the immunohistochemical expression of HER-2, EGFR, Cox-2 and Ki67 and its association with clinical-pathological parameters and overall survival. Our results found a concordance between the expression of HER-2 (K coefficient: 0.250), Cox-2 (K coefficient: 0.571), and Ki67 (K coefficient: 0.397) and a discordance between EGFR expression (K coefficient: -0.195) in primary mammary carcinomas and paired lymph node metastasis. Furthermore, a high Ki67 index (>24%), large tumor size and the presence of angiolymphatic invasion in canine primary mammary carcinoma with lymph node metastasis plus the presence of extracapsular extension in lymph nodes metastasis were also related to worse prognoses and shorter overall survival ( $P < 0.05$ ). In conclusion, our study demonstrates that primary mammary carcinomas with high expression of HER-2, Cox-2 and Ki67 also show high expression of these markers in paired lymph node metastasis. Moreover, the expression of these molecular markers in lymph nodes metastasis did not demonstrate a prognostic relevance.

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## 1. Introduction

As described in both canine and human mammary carcinomas, changes in genes encoding growth factors, growth factor receptors and other regulators of cellular proliferation might play a role in tumorigenesis and therapeutic response (Sorlie et al., 2001). Studies based on single molecular markers cannot accurately account for the heterogeneity of mammary carcinomas (Sorlie et al., 2001; Gama et al., 2008). Given the large number of cellular events involved in cell growth, differentiation, proliferation, invasion and metastasis, the investigation of multiple molecular alterations in conjunction has great relevance for the understanding of mammary carcinoma progression (Argyle and Khanna, 2013). Recently, the evaluation of molecular markers has been also applied to study of lymph nodes metastasis from human and canine mammary tumors (Cho et al., 2008; De Matos et al., 2007; Beha et al., 2012). As in women, regional lymph node status has a major impact on survival in dogs with mammary carcinomas (Hellmén et al., 1993; Yamagami et al., 1996; Chang et al., 2005; Araújo et al., 2015).

Mammary carcinomas can develop and progress by a stepwise accumulation of genetic changes, exposure to growth factors, or sex hormones. Among these genetic alterations, amplification of the *erbB* oncogenes, especially the human epidermal growth factor receptor genes *erbB-1* (EGFR, HER-1) and *erbB-2* (HER-2, *c-erb B 2*, HER-2/neu), have been shown to play a fundamental role in the progression of breast cancer and other solid tumors (Slamon et al., 1987; Bacus et al., 1994).

HER-2 gene amplification or HER-2 protein overexpression has been identified in approximately 30% of human breast cancers and it has been shown to be associated with poor prognosis and prediction of therapeutic response (Slamon et al., 1987; Vogel et al., 2002). In dogs, the prognostic relevance of HER-2 protein expression in mammary carcinomas has yet to be determined. Different authors have shown that the HER-2 protein overexpression in canine mammary carcinomas has been associated with worse prognosis; however, other studies have failed to demonstrate a prognostic relevance of HER-2 protein overexpression (De las Mulas et al., 2003; Hsu et al., 2009; Ressel et al., 2013).

EGFR has been expressed between 20% to 30% of human breast cancers and some studies have associated EGFR expression with poor clinical outcome (Aziz et al., 2002; Tsutsui et al., 2002). Elevated

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**Table 1**  
Details of immunohistochemical reagents and methods used in the study.

| Target antigen | Clone      | Manufacturer | Dilution | AR method                    | Retrieval solution (pH) | Incubation time (h) |
|----------------|------------|--------------|----------|------------------------------|-------------------------|---------------------|
| HER-2          | Polyclonal | Dako         | 1:200    | Water bath (98 °C)           | Citrate (6.0)           | 16                  |
| EGFR           | 31G7       | Zymed        | 1:50     | Enzymatic                    | Pepsin                  | 16                  |
| Cox-2          | SP21       | Labvision    | 1:80     | Water bath (98 °C)           | Citrate (6.0)           | 16                  |
| Ki67           | Mib-1      | Dako         | 1:25     | Pressurised heating (125 °C) | Citrate (6.0)           | 1                   |
| Cytokeratin    | AE1AE3     | Dako         | 1:100    | Water bath (98 °C)           | Citrate (6.0)           | 1                   |

HER-2: human epithelial grown factor receptor 2; EGFR: epithelial grown factor receptor; Cox-2: cyclooxygenase 2; AR: antigen retrieval.

EGFR serum levels and high expression have also been detected in canine mammary carcinomas using different methodologies, and its expression immunohistochemical has been associated with malignancy (Gama et al., 2009; Bertagnolli et al., 2011; Carvalho et al., 2013; Kim et al., 2013). However, the relevance of EGFR expression as an independent prognostic factor in human or dogs is still controversial (Rampaul et al., 2004; Kim et al., 2013).

Unlike HER-2 and EGFR, other molecules such as Cox-2 and Ki67 are well-recognized prognostic factors in most studies concerning canine mammary carcinomas. Cox-2 expression and a high cell proliferation index (Ki67) have been associated with disease progression, poor prognosis and a shorter survival time in dogs with mammary carcinomas (Dutra et al., 2008; Lavallo et al., 2009; Peña et al., 1998; Millanta et al., 2006; Queiroga et al., 2010; Santos et al., 2013). Nevertheless, data regarding the expression of these biomarkers in lymph node metastasis of canine mammary carcinomas are very limited (Pereira et al., 2009).

Due to the high incidence, clinical importance and new therapies targeted to canine mammary carcinomas, there is an increasing interest in prognostic and predictive biomarkers for the evaluation of tumor progression. In the present study, we evaluated the immunohistochemical expression of HER-2, EGFR, Cox-2 and Ki67 and its association with clinical-pathological parameters and overall survival in canine primary mammary carcinomas with and without metastasis and in paired lymph node metastasis.

## 2. Materials and methods

All procedures were performed under the guidelines and with the approval of the Ethics Committee in Animal Experimentation at the Federal University of Minas Gerais (UFMG), protocol 174/2011.

### 2.1. Samples

Canine mammary carcinomas were selected from the Veterinary School of the Federal University of Minas Gerais and the Laboratory of Comparative Pathology at the Institute of Biological Sciences at the Federal University of Minas Gerais. The cases were staged according to the tumor-node-metastasis (TNM) clinical staging system for canine mammary tumors: this system evaluates tumor size (T1, 0–3 cm; T2, 3–5 cm; T3, >5 cm); the involvement of regional lymph nodes (N0, no metastasis; N1, metastasis), and the presence of distant metastasis (M0, no metastasis; M1, metastasis). Cases were then categorized into five stages: I (T1N0M0); II (T2N0M0); III (T3N0M0); IV (T1,2,3N1M0); and V (T1,2,3N0,1M1) (Owen, 1980). The presence of lymph node metastasis was diagnosed through hematoxylin-eosin (H&E) routine staining and was confirmed by immunohistochemistry (IHC) (cytokeratin AE1/AE3 and/or vimentin). To rule out distant metastasis at time of diagnosis, all dogs were underwent thoracic radiography and abdominal ultrasound examination only when serum biochemical changes or increased abdominal size were observed. The cases that displayed systemic metastases were excluded, and 54 cases of primary mammary carcinomas with lymph node metastasis (N1) and 29 primary mammary carcinomas without lymph node metastasis (N0) were selected. Of

the 54 cases of primary mammary carcinomas with lymph node metastasis, a total of 99 lymph nodes were evaluated and diagnosed metastasis in 76 lymph nodes. However, in only 25 lymph nodes metastasis (of 25 cases) was possible an evaluation of the tumor markers.

### 2.2. Histopathology

All tumors and lymph node specimens were previously fixed in 10% neutral buffered formalin, paraffin-embedded and routinely prepared and stained with H&E. Tumors were classified according to the veterinary histological classification (Cassali et al., 2014; Misdorp et al., 1999). Furthermore, tumors were graded according to the Nottingham Grading System (Elston and Ellis, 1998) and the presence of angiolymphatic invasion (presence of neoplastic cells within the intratumoral and/or peritumoral angiolymphatic vessel spaces) was also evaluated. In the lymph node histological analysis, the presence and the number of lymph node affected with extracapsular extension (ECE) (the presence of neoplastic cells through the capsule of the lymph node metastasis into the perinodal tissue) were evaluated.

### 2.3. Immunohistochemistry

Consecutive 3 µm thick sections of primary mammary carcinomas (N0 and N1) and lymph nodes metastasis were cut for IHC analysis, and the antigen was immunodetected using the Dako Advance (HRP) Visualization Method (Dako, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methyl alcohol. The reagents were applied manually and immunoreactivity was visualized with the chromogen diaminobenzidine (DAB Substrate System, Dako, Carpinteria, CA, USA). Details of the antibodies, dilutions, antigen retrieval procedures, and incubation times used are provided in Table 1. The cytokeratin (CK) AE1/AE3 was used to confirm the presence of metastasis. Sections from the HER-2, EGFR and Cox-2, positive canine mammary carcinoma were used as positive controls. Negative controls were assessed using normal serum as the primary antibody.

HER-2 expression were determined by a scoring system established by the American Society of Clinical Oncology, College of American Pathologists (ASCO/CAP) (Wolff et al., 2013) (0 = no membrane staining or incomplete and faint/barely perceptible membrane staining in ≤10% of tumor cells; 1 + = incomplete and faint/barely perceptible membrane staining in ≥10% of tumor cells; 2 + = incomplete and/or weak/moderate membrane staining in >10% of tumor cells or complete and intense membrane staining in ≤10% of tumor cells; and 3 + = complete and intense membrane staining in >10% of tumor cells). In our study, specimens with scores of 0, 1 + and 2 + were regarded as negative, and score of +3 were defined as positive.

EGFR expression was evaluated by adapted criteria HER-2 assessment (Wolff et al., 2013) and specimens with scores of 0, 1 + and 2 + were regarded as negative, and score of 3 + were defined as positive.

Positivity for Cox-2 was indicated by cytoplasmic staining. The number of Cox-2 positive cells was evaluated semi-quantitatively,

with the distribution score defined by the estimated percentage of positive cells in 5 fields at 400× magnification: 0 = absent, 1 ≤ 10% of cells were stained, 2 = between 10% and 30% of cells were stained, 3 = between 31% and 60% of cells were stained, 4 = >61% of cells were stained. To evaluate staining intensity, values from 0 to 3 were

attributed: 0 = absence (–), 1 = weak staining (+), 2 = moderate staining (++) and 3 = strong staining (+++). The distribution score and intensity were multiplied to obtain a total score, which ranged from 0 to 12, with scores from 0 to 5 considered low and scores from 6 to 12 considered high (Lavalle et al., 2009).

**Table 2**

Clinical-pathological parameters of the primary mammary carcinomas with lymph node metastasis and without metastasis.

|   | n (%) | Primary mammary carcinomas with lymph node metastasis <sup>a</sup> | Primary mammary carcinomas without metastasis <sup>b</sup> |
|---|-------|--|--|
| <i>Age (mean ± SD)</i>                  | 79    | 10.88 ± 2.7  | 10.51 ± 2.9  |
| <i>Breed</i>                            |       |  |  |
| Crossbreed                              | 21    | 17 (81)  | 4 (19)   |
| Pure breeds <sup>c</sup>                | 58    | 34 (58.6)  | 24 (41.4)  |
| <i>Reproductive status*</i>             |       |  |  |
| Intact                                  | 47    | 27 (57.4)  | 20 (42.6)  |
| Spayed                                  | 21    | 19 (90.5)  | 2 (9.5)  |
| <i>Tumor localization*</i>              |       |  |  |
| Thoracic                                | 3     | 3 (100)  | 0 (0)  |
| Cranial abdominal                       | 12    | 11 (91.7)  | 1 (8.3)  |
| Caudal abdominal                        | 25    | 12 (48)  | 13 (52)  |
| Inguinal                                | 28    | 16 (57.1)  | 12 (42.9)  |
| Multicentric                            | 12    | 10 (83.3)  | 2 (16.7)   |
| <i>Histological type*</i>               |       |  |  |
| Mixed tumor carcinoma                   | 32    | 11 (34.4)  | 21 (65.6)  |
| Tubular carcinoma                       | 3     | 3 (100)  | 0 (0)  |
| Papillar carcinoma                      | 11    | 8 (72.7)   | 3 (27.3)   |
| Solid carcinoma                         | 17    | 13 (76.5)  | 4 (23.5)   |
| Micropapillary carcinoma                | 12    | 12 (100)   | 0 (0)  |
| Special types of carcinoma <sup>d</sup> | 2     | 2 (100)  | 0 (0)  |
| Carcinosarcoma                          | 6     | 5 (83.3)   | 1 (16.7)   |
| <i>Histological grade*<sup>e</sup></i>  |       |  |  |
| I                                       | 19    | 7 (36.8)   | 12 (63.2)  |
| II                                      | 44    | 29 (65.9)  | 15 (34.1)  |
| III                                     | 19    | 17 (89.5)  | 2 (10.5)   |
| <i>Tumor size</i>                       |       |  |  |
| T1 (<3 cm)                              | 25    | 12 (48)  | 13 (52)  |
| T2 (>3 and <5 cm)                       | 23    | 14 (60.9)  | 9 (39.1)   |
| T3 (>5 cm)                              | 31    | 24 (77.4)  | 7 (22.6)   |
| <i>Stage (TNM)*<sup>f</sup></i>         |       |  |  |
| I                                       | 13    | 0 (0)  | 13 (100)   |
| II                                      | 9     | 0 (0)  | 9 (100)  |
| III                                     | 7     | 0 (0)  | 7 (100)  |
| IV                                      | 54    | 54 (100)   | 0 (0)  |
| <i>Angiolymphatic invasion*</i>         |       |  |  |
| Present                                 | 27    | 22 (81.5)  | 5 (18.5)   |
| Absent                                  | 55    | 31 (56.4)  | 24 (43.6)  |
| <i>Extracapsular extension (ECE)</i>    |       |  |  |
| Present                                 | 22    | 22 (100)   | NA   |
| Absent                                  | 32    | 32 (100)   |  |
| <i>Number of lymph nodes with ECE</i>   |       |  |  |
| 1                                       | 15    | 15 (100)   | NA   |
| ≥2                                      | 7     | 7 (100)  |  |

ECE: extracapsular extension; NA: not applied.

<sup>a</sup> Some data were not available or an evaluation was not possible for all 54 primary mammary carcinomas with lymph node metastasis.

<sup>b</sup> Some data were not available for all 29 primary mammary carcinomas without metastasis.

<sup>c</sup> Pure breeds included Cocker Spaniel, Dachshund, Pitbull, German Shepherd, Pinscher, Schnauzer, Basset Hound, Bichon Frise, Brazilian Fila, Golden Retriever, Siberian Husky and Yorkshire.

<sup>d</sup> Special types of carcinoma included rich in lipids carcinoma and pleomorphic lobular carcinoma.

<sup>e</sup> Histological grading based on that of Elston and Ellis (1998).

<sup>f</sup> I (T1N0M0), II (T2N0M0), III (T3N0M0) and IV (T1,2,3N1M0).

\*  $p < 0.05$  significant difference in Exact of Fisher or Chi-square test.

The cell proliferative index was calculated by counting the number of nuclei positive for Ki-67 staining in a total of 1000 neoplastic cells from each lesion in hot spot areas (Dutra et al., 2008). A cut-off of 24% positive neoplastic cells for the analysis of Ki67 was used (Peña et al., 1998), with >24% Ki67 index considered to be a high cell proliferation index and <24% Ki67 index considered to be a low cell proliferation index.

**2.4. Overall survival**

The overall survival was defined as the period (in days) between surgical tumor removal and death due to the tumor. The follow-up period was 1370 days. Animals that died from unknown causes or from causes unrelated to the mammary tumor were censored.

**2.5. Statistical analysis**

Statistical analysis was performed with the software GraphPad Prism 6.0. The D'Agostino & Pearson omnibus normality test was used to determine if the data were normally distributed. The Mann-Whitney and unpaired t tests were used to compare the Ki67 index means and size tumor. Possible correlations were investigated by Spearman tests and the associations were described by Chi-square and Fisher's exact tests. The survival curves were calculated with the Kaplan-Meier estimate using the log-rank test. Values were considered statistically significant when  $P < 0.05$ .

The agreement between the HER-2, EGFR, Cox-2 and Ki67 status of primary tumors and paired lymph node metastasis was expressed by the Cohen Kappa coefficient using the software GraphPad Prism QuickCalcs. The relation between the values and the levels of agreement

**Table 3**

Immunohistochemical data of primary mammary carcinomas without metastasis, primary mammary carcinomas with lymph node metastasis and lymph node metastasis.

|                          | Primary mammary carcinomas without metastasis<br>n/total (%) <sup>a</sup> | Primary mammary carcinomas with lymph node metastasis<br>n/total (%) <sup>b</sup> | Lymph nodes metastasis<br>n/total (%) <sup>c</sup> |
|--------------------------|---|---|--|
| <i>HER-2<sup>d</sup></i> |   |   |  |
| Negative                 | 23/25 (88)  | 44/48 (91.7)  | 15/23 (65.2)                                       |
| Positive                 | 3/25 (12)   | 4/48 (8.3)*   | 8/23 (34.8)*                                       |
| <i>EGFR<sup>e</sup></i>  |   |   |  |
| Negative                 | 22/26 (84.6)  | 42/48 (87.5)  | 22/25 (88)   |
| Positive                 | 4/26 (15.4)   | 6/48 (12.5)   | 3/25 (12)  |
| <i>Cox-2<sup>f</sup></i> |   |   |  |
| Low score                | 21/23 (91.3)  | 43/50 (86)  | 19/24 (79.2)                                       |
| High score               | 2/23 (8.7)  | 7/50 (14)   | 5/24 (20.8)  |
| <i>Ki67 index</i>        |   |   |  |
| <24%                     | 15/21 (71.4)  | 24/48 (50)  | 9/19 (47.4)  |
| >24%                     | 6/21 (28.6)   | 24/48 (50)  | 10/19 (52.6)                                       |

<sup>a</sup> Evaluation of HER-2, EGFR, Cox-2 and Ki67 staining was not possible in all 29 primary mammary carcinomas without metastasis.

<sup>b</sup> Evaluation of HER-2, EGFR, Cox-2 and Ki67 staining was not possible in all 54 primary mammary carcinomas with lymph node metastasis.

<sup>c</sup> Evaluation of HER-2, EGFR, Cox-2 and Ki67 staining was not possible in all 25 lymph node metastasis.

<sup>d</sup> Negative: scores 0, 1+ and 2+/Positive: score 3+.

<sup>e</sup> Negative: scores 0, 1+ and 2+/Positive: score 3+.

<sup>f</sup> Low score: score 0 to 5/High score: score 6 to 12.

\* Significant difference ( $p = 0.01$ ) in Exact of Fisher test between primary mammary carcinomas and lymph node metastasis.

were those used by Landis and Koch (1977) with values of 0.00–0.20 indicating slight agreement, values of 0.21–0.40 indicating fair agreement, values of 0.41–0.60 indicating moderate agreement, values of 0.61–0.80 indicating substantial agreement, and values of 0.81–1.00 indicating almost perfect agreement.

### 3. Results

#### 3.1. Clinical-pathological parameters in canine primary mammary carcinomas and regional lymph nodes

The clinical-pathological features of the animals with primary mammary carcinoma without metastasis (N0) and with lymph node metastasis (N1) are summarized in Table 2. The ages of the animals at the time of surgery ranged from 4 to 16 years with a mean of  $10.88 \pm 2.7$  years and  $10.51 \pm 2.9$  years in dogs with lymph node metastasis and without metastasis, respectively ( $P > 0.05$ ).

The anatomic location of the carcinomas was more frequent in inguinal glands in dogs with lymph node metastasis (16/28, 57.1%) and in caudal abdominal glands in dogs without metastasis (13/25, 52%) ( $P = 0.02$ ). The thoracic location was observed only in three primary mammary carcinomas with lymph node metastasis.

Higher frequency of carcinoma in mixed tumors (21/32, 65.6%) in dogs without metastasis and of non-mixed tumors and carcinosarcomas (43/51, 84.3%) in dogs with lymph node metastasis ( $P < 0.001$ ) were observed. Grade I (12/19, 63.2%) was more predominant in primary mammary carcinomas without metastasis and grade II (29/44, 65.9%) and grade III (17/19, 89.5%) were more predominant in primary mammary carcinomas with lymph node metastasis ( $P < 0.01$ ).

The tumor size ranged from 0.5 to 10 cm (mean of  $3.62 \pm 2.49$  cm) in primary mammary carcinoma without metastasis and from 0.4 to 15 cm (mean of  $5.39 \pm 3.53$  cm) in primary mammary carcinoma with lymph node metastasis ( $P = 0.02$ ). Angiolymphatic invasion (22/27, 81.5%) was more frequent in primary mammary carcinoma with lymph node metastasis than primary mammary carcinoma without metastasis ( $P < 0.01$ ).

In the lymph node histological analysis, ECE was identified in 42.1% (32/76) of the lymph nodes metastasis. Additionally, ECE was observed in 40.74% (22/54) of cases of primary mammary carcinomas studied. In 68.2% (15/22) and in 31.8% (7/22) of these cases, one lymph node and  $\geq 2$  lymph nodes with ECE, respectively, were observed.

In primary mammary carcinoma without metastasis, high histological grade correlated with larger tumors ( $r = 0.45$ ,  $P = 0.01$ ) and with the presence of angiolymphatic invasion ( $r = 0.41$ ,  $P = 0.02$ ). Furthermore, the presence of angiolymphatic invasion was correlated with larger tumors ( $r = 0.47$ ,  $P = 0.01$ ).

In primary mammary carcinoma with lymph node metastasis, the presence of angiolymphatic invasion was correlated with high histological grade ( $r = 0.40$ ,  $P = 0.003$ ), with the presence of ECE ( $r = 0.53$ ,  $p < 0.001$ ) and with a high number of lymph nodes with ECE ( $r = 0.48$ ,  $P < 0.001$ ).

#### 3.2. Molecular parameters in primary mammary carcinomas with lymph node metastasis and without metastasis

The immunohistochemical data of the primary mammary carcinomas with lymph node metastasis and without metastasis are summarized in Table 3. There was no significant difference in the frequency of the expression of all markers between primary mammary carcinomas with lymph node metastasis and without metastasis. The mean expression of the Ki67 index in primary mammary carcinomas with lymph node metastasis and without metastasis was  $26.33 \pm 19.80\%$  and  $19.27 \pm 22.03\%$ , respectively ( $P > 0.05$ ).

In primary mammary carcinomas with lymph node metastasis, the HER-2-positive tumors were positively correlated with a high cell proliferation index ( $r = 0.32$ ;  $P = 0.03$ ).

#### 3.3. Relationship between the molecular parameters in primary mammary carcinomas and in lymph node metastasis

The lymph node metastasis immunohistochemical data are also summarized in Table 3. A higher frequency of positivity of HER-2 in lymph node metastasis was observed in relation to primary mammary carcinomas ( $P = 0.01$ ).

The mean expression of Ki67 index in primary mammary carcinomas and in lymph node metastasis was of  $26.33 \pm 19.80\%$  and  $28.91 \pm 20.79\%$ , respectively ( $P > 0.05$ ).

The HER-2, EGFR, Cox-2 and Ki67 status of the primary mammary carcinomas and paired lymph nodes metastasis are shown in Table 4. The HER-2 status of the primary mammary carcinomas and paired lymph nodes metastasis was concordant in 15 of 21 cases (71.4%), including 2 (9.5%) with positivity to HER-2 and 13 (61.9%) with negativity to this marker. The Cohen K coefficient was 0.25, indicating fair agreement. The EGFR status agreed in 14 of 21 cases (66.7%), all with negativity to EGFR. However, the Cohen K coefficient was  $-0.195$ , indicating discordance. The Cox-2 status agreed in 17 of 20 cases (85%), including 3 (15%) with high score and 14 (75%) with low score. The Cohen K coefficient was 0.571, indicating moderate agreement. Finally, Ki67 status agreed in 12 of 17 cases (70.6%), including 8 (47.1%) with a high cell proliferation index and 4 (23.5%) with a low cell proliferation index. The Cohen K coefficient was 0.397, indicating fair agreement.

Additionally, in the correlation analysis, high Cox-2 score in primary mammary carcinomas correlated with high Cox-2 score in lymph nodes metastasis ( $r = 0.57$ ,  $P = 0.008$ ).

The Figs. 1 to 8 illustrating our immunohistochemical results in primary mammary carcinomas and lymph nodes metastasis.

#### 3.4. Relationship between clinical-pathological and molecular parameters

In primary mammary carcinomas without metastasis, high histological grade correlated with a high cell proliferation index ( $r = 0.60$ ,  $P = 0.004$ ) and positivity to HER-2 ( $r = 0.50$ ,  $P = 0.008$ ). Furthermore, positivity to EGFR correlated with the presence of angiolymphatic invasion ( $r = 0.40$ ,  $P = 0.03$ ) and with larger tumors ( $r = 0.52$ ,  $P = 0.007$ ).

In primary mammary carcinomas with lymph node metastasis, a high cell proliferation index correlated with presence of ECE ( $r = 0.37$ ,  $P = 0.008$ ) and a high number of lymph nodes with ECE ( $r = 0.39$ ,  $P = 0.006$ ).

#### 3.5. Overall survival

Survival data were available for 78 dogs. In total, 33 (42.3%) dogs died because of mammary carcinoma, 5 of which were euthanised because of the disease. Twenty eight (35.9%) dogs were alive up between 152 and 1095 days post-surgery and 17 (21.8%) dogs died from causes unrelated to the mammary tumor between 27 and 1370 days post-surgery.

The mean overall survival of dogs with primary mammary carcinoma with lymph node metastasis and without metastasis was  $351.56 \pm 272.17$  days and  $552.04 \pm 327.79$  days ( $P = 0.004$ ), respectively. The log rank test showed longer overall survival of dogs with primary mammary carcinoma without metastasis (not reached the median survival time) than those with lymph node metastasis (median 395 days) ( $P = 0.002$ ) (Fig. 9).

In dogs with primary mammary carcinoma with lymph node metastasis, shorter survival was correlated with high histological grade ( $r = -0.32$ ,  $P = 0.02$ ), the presence of angiolymphatic invasion ( $r = -0.29$ ,  $P = 0.04$ ), the presence of ECE ( $r = -0.55$ ,  $P < 0.001$ ) and high number of lymph nodes with ECE ( $r = -0.56$ ,  $P < 0.001$ ). However, no correlation between overall survival and clinical pathological parameters was observed in dogs with primary mammary carcinoma without metastasis.

**Table 4**

HER-2, EGFR, Cox-2 and Ki67 index status in primary mammary carcinomas and paired lymph node metastasis.

| Lymph node metastasis n (%) <sup>a</sup> | HER-2 <sup>b</sup> |           | EGFR <sup>c</sup> |           | Cox-2 <sup>d</sup> |            | Ki67 index <sup>e</sup> |          |
|--|--------------------|-----------|-------------------|-----------|--------------------|------------|-------------------------|----------|
|  | Positive           | Negative  | Positive          | Negative  | Low score          | High score | <24%                    | >24%     |
| Mammary carcinomas                       |                    |           |                   |           |                    |            |                         |          |
| n (%) <sup>a</sup>                       |                    |           |                   |           |                    |            |                         |          |
| HER-2 <sup>b</sup>                       |                    |           |                   |           |                    |            |                         |          |
| Positive                                 | 2 (9.5)            | 1 (4.8)   |                   |           |                    |            |                         |          |
| Negative                                 | 5 (23.8)           | 13 (61.9) |                   |           |                    |            |                         |          |
| EGFR <sup>c</sup>                        |                    |           |                   |           |                    |            |                         |          |
| Positive                                 |                    |           | 0 (0)             | 4 (19)    |                    |            |                         |          |
| Negative                                 |                    |           | 3 (14.3)          | 14 (66.7) |                    |            |                         |          |
| Cox-2 <sup>d</sup>                       |                    |           |                   |           |                    |            |                         |          |
| Low score                                |                    |           |                   |           | 14 (70)            | 2 (10)     |                         |          |
| High score                               |                    |           |                   |           | 1 (5)              | 3 (15)     |                         |          |
| Ki67 index <sup>e</sup>                  |                    |           |                   |           |                    |            |                         |          |
| <24%                                     |                    |           |                   |           |                    |            | 4 (23.5)                | 1 (5.9)  |
| >24%                                     |                    |           |                   |           |                    |            | 4 (23.5)                | 8 (47.1) |

<sup>a</sup> The evaluation of HER-2, EGFR, Cox-2 and Ki67 status in primary mammary carcinomas and paired lymph node metastasis not was possible in all 25 cases.

<sup>b</sup> K coefficient: 0.250 (95% confidence interval: -0.158 to 0.658).

<sup>c</sup> K coefficient: -0.195 (95% confidence interval: -0.345 to -0.046).

<sup>d</sup> K coefficient: 0.571 (95% confidence interval: 0.142 to 1.000).

<sup>e</sup> K coefficient: 0.397 (95% confidence interval: -0.015 to 0.809).

In the survival curve analysis, a statistically significant difference in overall survival was observed with histological grade in dogs with primary mammary carcinoma without metastasis, with tumor size and ECE in dogs with primary mammary carcinoma with lymph node metastasis, and with angiolymphatic invasion in dogs with primary mammary carcinoma with lymph node metastasis and without metastasis.

When evaluated the primary mammary carcinomas with lymph node metastasis, a shorter overall survival was observed in dogs with T2 tumors (median 275 days,  $P = 0.02$ ) (Fig. 10), with the presence of angiolymphatic invasion (median 188 days,  $P = 0.01$ ) (Fig. 11) and with the presence of ECE (median 180 days,  $P < 0.0001$ ) (Fig. 12).

A poor clinical outcome was associated with the presence of angiolymphatic invasion (median 341 days,  $P = 0.008$ ) (Fig. 13) and with grade III tumors (median 263 days;  $P = 0.005$ ) in dogs with primary mammary carcinomas without metastasis (Fig. 14).

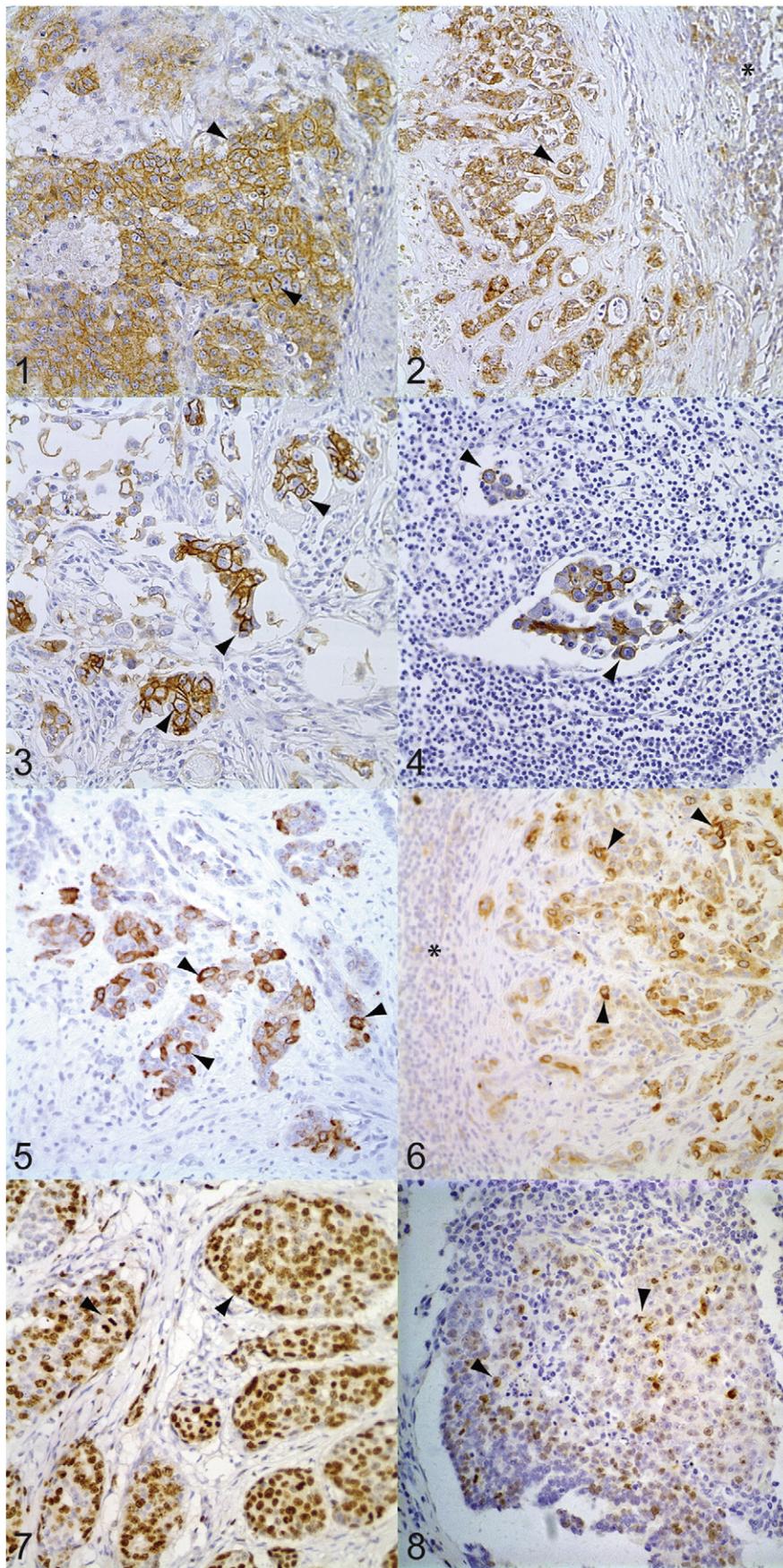
There were not significant correlations between overall survival and tumor markers expression in dogs with primary mammary carcinomas with lymph node metastasis and without metastasis. These results were confirmed in survival curve analyses ( $P > 0.05$ ). However, a shorter overall survival of animals with high proliferative tumors (median 322 days) in relation to animals with a low proliferative tumors (not reached the median survival time) was observed in primary mammary carcinomas with lymph node metastasis ( $P = 0.03$ ) (Fig. 15). Additionally, when the expression of HER-2, EGFR, Cox-2 and Ki67 was evaluated in lymph node metastasis, no significant difference was observed in the overall survival curve analysis ( $P > 0.05$ ).

#### 4. Discussion

Primary mammary carcinomas with metastasis have a significant up-regulation of genes associated with cell cycle regulation, matrix modulation, protein folding and proteasomal degradation, and down-regulation of differentiation genes, growth factor pathway genes and regulators of actin organization (Klopfleisch et al., 2010). In our study, the frequency of expression of HER-2, EGFR, Cox-2 and Ki67 did not differ significantly between primary mammary carcinomas with lymph node metastasis and without metastasis.

Some studies have demonstrated that the high frequency of HER-2 protein overexpression in in situ ductal carcinomas of the breast could be an early event in carcinogenesis (Xu et al., 2002). This fact can explain the similar frequency of this marker in the invasive primary carcinomas studied (with lymph node metastasis and without metastasis). However, lymph nodes metastasis (34.8%) had a significantly higher frequency of positivity to HER-2 compared to primary mammary carcinomas (8.3%). The epithelial phenotype is an event necessary in the formation of secondary tumors in lymph nodes (Hugo et al., 2007). HER-2 expression occurs mainly in neoplastic epithelial cells in a variety of solid tumors, including mammary tumors (Slamon et al., 1987; Bacus et al., 1994). Furthermore, one study demonstrated a correlation between the phenomenon of epithelial to mesenchymal transition in breast cancer and triple-negative tumors (HER-2 negative, estrogen and progesterone receptors negative) (Jeong et al., 2012). Therefore, our findings may suggest an important role of HER-2 overexpression in the mechanism related to the implantation of neoplastic cells in metastatic niches, such as the mesenchymal to epithelial transition in canine mammary tumors. Moreover, the biologic phenomenon of tumor heterogeneity described in breast cancer could explain the selection of HER-2-positive subclones in the metastasis observed in canine mammary tumors (Malinowsky et al., 2012; Niikura et al., 2012).

Another marker of the same family of human epidermal growth factor receptors, EGFR was not associated with survival in the present study or in the literature (Gama et al., 2009). In our series, we did not find a significant difference in the overall survival of dogs with positivity or negativity for EGFR when evaluated primary mammary carcinomas with lymph node metastasis, primary mammary carcinomas without metastasis and lymph nodes metastasis. Furthermore, the frequency of EGFR expression did not differ between primary mammary carcinomas with lymph node metastasis and without metastasis. EGFR expression has been suggested to be an early event in the carcinogenesis of canine mammary carcinoma, which could explain the findings of the present study (Bertagnolli et al., 2011; Ferreira et al., 2012; Silva et al., 2014). However, an association between the positivity to EGFR and the presence of lymph node metastasis was observed by Guimarães et al. (2014) but not by Gama et al. (2009). These conflicting results may be related to the different analytic methods for evaluating EGFR expression in these studies. In our study, positivity to EGFR was correlated with the



presence of angiolymphatic invasion and larger tumors in primary mammary carcinomas without metastasis. These findings are consistent with previous studies that found a correlation between positivity to EGFR and factors related to biologic aggressiveness such as larger tumors, high grade histologic, the presence of necrosis and the presence of lymphatic invasion (Carvalho et al., 2013; Gama et al., 2009; Kim et al., 2013; Guimarães et al., 2014).

To our knowledge, the present study is the first description of the overall survival of dogs with metastatic carcinoma according to Cox-2 expression in lymph node metastasis. Interestingly, a positive correlation between Cox-2 expression in primary tumors and lymph node metastasis was observed. However, in our study, there was not a significant difference in the frequency of the expression of Cox-2 in primary mammary carcinomas with lymph node metastasis and without metastasis. Few studies have found that the high expression of Cox-2 is associated with the presence of lymph node metastasis in dogs (Guimarães et al., 2014; Queiroga et al., 2010). An analysis of Cox-2 expression in canine metastatic lesions was presented only by Pereira et al. (2009). These authors found that in almost all cases, the labeling intensity of metastatic lesions (pulmonary and lymph node metastasis) was similar or stronger than that of the primary, indicating that Cox-2 metabolic products contributing to metastatic spread of neoplastic cells.

Additionally, the present study did not demonstrate the prognostic relevance of Cox-2 expression when evaluated primary mammary carcinomas with lymph node metastasis, primary mammary carcinomas without metastasis and lymph nodes metastasis. Although the role of Cox-2 and its derived metabolites in tumorigenesis appears to be clear, the prognostic relevance of Cox-2 expression in cancer has not been clearly established (Soslow et al., 2000; Surh et al., 2001; Lavallo et al., 2009). Some studies in veterinary medicine have found that dogs with mammary carcinoma and high Cox-2 expression have a shorter survival time (Millanta et al., 2006; Lavallo et al., 2009; Queiroga et al., 2010). In the current study, only 6 dogs with primary mammary carcinomas with lymph node metastasis had high Cox-2 score, which could explain our findings. Thus, the evaluation of Cox-2 in canine primary tumors and metastases should be investigated in additional studies using different molecular techniques.

In the present study, we demonstrated that Ki67 have prognostic relevance in dogs with primary mammary carcinomas with lymph node metastasis. A shorter overall survival of dogs with a high Ki67 index was also described in previous studies (Peña et al., 1998; Santos et al., 2013). Some multivariable studies, however, reported contrasting results, which may be explained by the fewer number of malignant tumors included in these studies (Löhr et al., 1997; Lee et al., 2004). In our series, a fair agreement between primary mammary carcinomas and paired lymph node metastasis was observed, and most of the concordant cases had high Ki67 index. Interestingly, 23.5% of the cases had a high cell proliferation index in the primary carcinomas but not in lymph node metastasis and 5.9% of the cases had a high cell proliferation index in lymph node metastasis but not in primary carcinomas. Using a cut-off of 24% for Ki67 was not observed significant difference in the frequency between primary mammary carcinomas with lymph node metastasis and without metastasis. Additionally, the frequency also did not differ between the primary carcinomas and lymph node metastasis. Thus, our results may suggest that the primary mammary carcinoma and paired lymph node metastasis are in general made up of cell populations with similar characteristics. Changes in the proliferative

activity may occur during growth as a result of the selection of subpopulations with a high metastatic potential, due to the heterogeneity of the primary tumor, genotypic instability, or clonal proliferation during the process of metastasis (Kakeji et al., 1991). Furthermore, the association between the Ki67 index and the presence of lymph node metastasis is controversial in human and canine studies of mammary tumors (De Matos et al., 2006; Leonardi et al., 1992; Peña et al., 1998).

Interesting, in the current study, a significant correlation between a high Ki67 index in primary mammary carcinomas with lymph node metastasis and the presence of ECE and a high number of lymph nodes with ECE was found. Furthermore, dogs presenting lymph nodes with ECE had shorter overall survival in relation to those without ECE. The evaluation of extracapsular extension in the lymph nodes metastasis of dogs with mammary carcinomas is a novel aspect of the present study. This parameter has been studied in human breast cancer and its prognostic relevance has been proven (Yajima et al., 2015). In canine mammary tumors, ECE has not previously been described and our findings suggest that this feature could indicate more extensive disease and may explain the lower survival of these dogs.

## 5. Conclusions

Our study demonstrates that primary mammary carcinomas with lymph node metastasis, presenting a high expression of HER-2, Cox-2 and Ki67 also have a high expression of these markers in paired lymph node metastasis. However, higher frequency of positivity to HER-2 in lymph node metastasis than in primary carcinomas was observed. Furthermore, a high Ki67 index, larger tumor size and the presence of angiolymphatic invasion in canine primary mammary carcinomas with lymph node metastasis and the occurrence of ECE in lymph nodes metastasis were also related to poor prognosis and shorter overall survival. Thus, our results suggest that the molecular parameters studied are associated or correlated with tumor aggressiveness and may be considered in future studies of canine mammary tumors and applied in clinical management.

## Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

## Acknowledgements

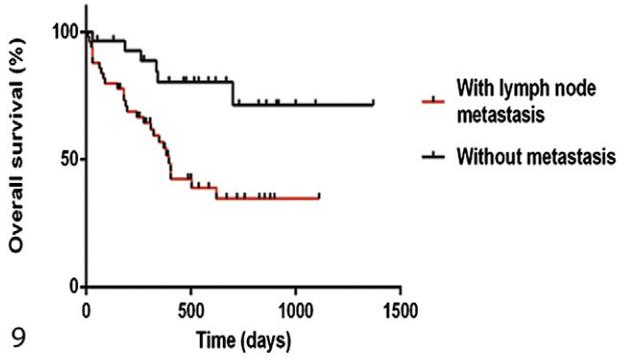
This work was financially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (470696/2011-7), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG), Brazil.

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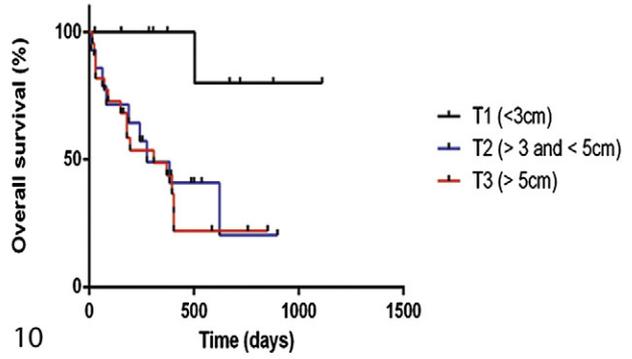
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**Fig. 1.** Canine mammary gland, solid carcinoma: malignant epithelial component shows intense membrane staining for HER2 in >10% of tumor cells (3+ score). Immunohistochemistry (IHC). 400×. **Fig. 2.** Canine lymph node, metastasis of micropapillary carcinoma: intense membrane staining for HER-2 in >10% of epithelial tumor cells replacing part of the lymphoid parenchyma (asterisk). IHC. 400×. **Fig. 3.** Canine mammary gland, Micropapillary carcinoma: malignant epithelial component shows intense membrane staining for EGFR in >10% of tumor cells (3+ score). IHC. 400×. **Fig. 4.** Canine lymph node, metastasis of micropapillary carcinoma: intense membrane staining for HER-2 in >10% of epithelial tumor cells distributed in the medular lymphatic sinuses. IHC. 400×. **Fig. 5.** Canine mammary gland, tubular carcinoma: malignant epithelial component show intense cytoplasm staining for Cox-2 between 31% and 60% of tumor cells (high score). IHC. 400×. **Fig. 6.** Canine lymph node, metastasis of tubular carcinoma: intense cytoplasm staining for Cox-2 between 31% and 60% of tumor cells (high score) replacing part of the lymphoid parenchyma (asterisk). IHC. 400×. **Fig. 7.** Canine mammary gland, solid carcinoma: malignant epithelial component shows nuclear staining for Ki67 in >24% of tumor cells (high proliferation index). IHC. 400×. **Fig. 8.** Canine lymph node, metastasis of solid carcinoma: nuclear staining for Ki67 in >24% of epithelial tumor cells (high proliferation index) distributed in the lymphoid parenchyma. IHC. 400×.

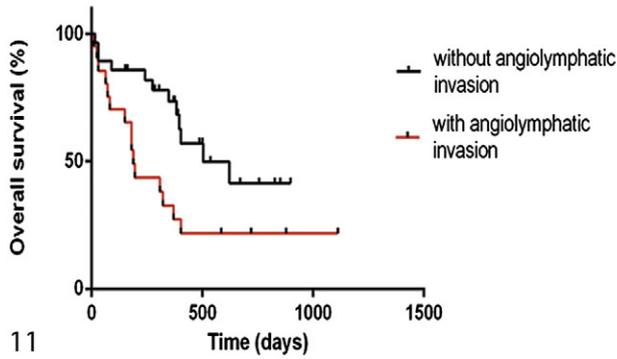
Primary mammary carcinoma



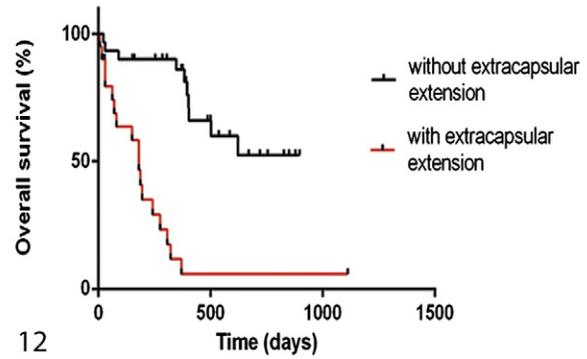
Primary mammary carcinoma with lymph node metastasis



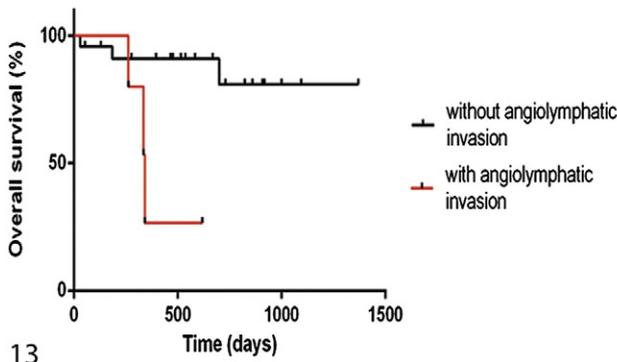
Primary mammary carcinoma with lymph node metastasis



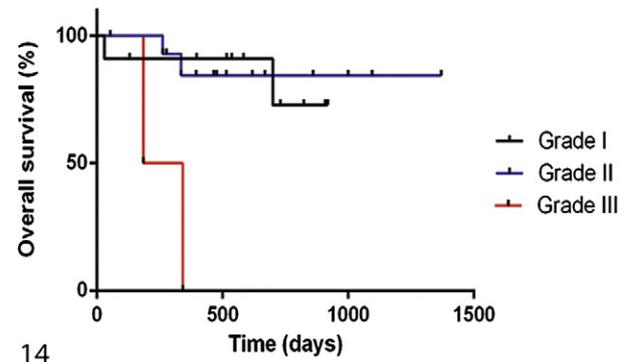
Primary mammary carcinoma with lymph node metastasis



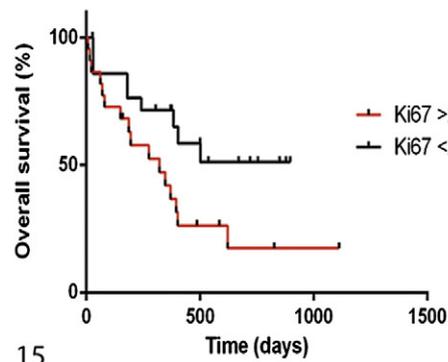
Primary mammary carcinoma without metastasis



Primary mammary carcinoma without metastasis



Primary mammary carcinoma with lymph node metastasis



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**Fig. 9.** Kaplan-Meier survival curve for the animals with primary mammary carcinoma with lymph node metastasis ( $n = 50$ , median, 395 days) and without metastasis ( $n = 28$ , not reaching median survival) ( $P = 0.002$ ). **Fig. 10:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma with lymph node metastasis according to size tumor: T1 (<3 cm,  $n = 11$ , not reaching median survival), T2 (>3 and <5 cm,  $n = 14$ ; median, 275 days) and T3 (>5 cm,  $n = 22$ , median, 308 days) ( $P < 0.02$ ). **Fig. 11:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma with lymph node metastasis according to angiolymphatic invasion: without angiolymphatic invasion ( $n = 28$ , median, 503 days) and with angiolymphatic invasion ( $n = 21$ , median, 188 days) ( $P = 0.01$ ). **Fig. 12:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma with lymph node metastasis according to extracapsular extension: without extracapsular extension ( $n = 30$ , not reaching median survival) and with extracapsular extension ( $n = 20$ , median, 180 days) ( $P < 0.0001$ ). **Fig. 13:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma without metastasis according to angiolymphatic invasion: without angiolymphatic invasion ( $n = 23$ ; not reaching median survival) and with angiolymphatic invasion ( $n = 5$ , median, 341 days) ( $P = 0.008$ ). **Fig. 14:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma without metastasis according to histologic grade: grade I ( $n = 11$ , not reaching median survival), grade II ( $n = 15$ , not reaching median survival) and grade III ( $n = 2$ , median 263 days) ( $P = 0.005$ ). **Fig. 15:** Kaplan-Meier survival curve for the animals with primary mammary carcinoma with lymph node metastasis according to the Ki67 index: Ki67 > 24% index ( $n = 22$ , median 322 days) and Ki67 < 24% index ( $n = 22$ , not reaching median survival) ( $P = 0.03$ ).

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